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Mechanisms in Experimental Venous Valve Failure and their Modification by Daflon© 500 mg

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Objectives. *To characterize the acute response of the vein wall to venous hypertension and associated altered fluid shear stress and to test the effect of micronized purified flavonoid fraction (MPFF, Daflon® 500), on this response.*
Material and methods. *A femoral arteriovenous fistula was created in Wistar rats (n = 48). A cohort of 24 rats received oral treatment with MPFF (100 mg/kg/day body weight), 24 rats underwent the arteriovenous fistula procedure and received no treatment. At days 1, 7 and 21 the animals (n = 8 at each time point) were killed. Experimental parameters measured included limb circumference, blood flow at the sapheno-femoral junction, leukocyte infiltration and gelatinase activity (matrix metalloproteinase, MMP).*
Results. *The acute rise in venous hypertension was accompanied by limb edema and venous reflux together with an eventual loss of valve leaflets in the saphenous vein. There was an increase in granulocyte and macrophage infiltration into the venous wall and the surrounding tissue, and a lesser increase in T- and B-lymphocyte infiltration. These changes were accompanied by a local increase in the proteolytic enzymes, MMP-2 and MMP-9. Administration of MPFF reduced the edema and lessened the venous reflux produced by the acute arteriovenous fistula. Decreased levels of granulocyte and macrophage infiltration into the valves were also observed compared with untreated animals.*
Conclusions. *Venous hypertension caused by an arteriovenous fistula resulted in the development of venous reflux and an inflammatory reaction in venous valves culminating in their destruction. MPFF was able to delay the development of reflux and suppress damage to the valve structures in this rat model of venous hypertension.*

Keywords: Venous insufficiency; Inflammation; Matrix metalloproteinase; Leucocytes; Monocytes; Lymphocytes.

Introduction

Histological and immuno-cytochemical analyses of venous valves and the venous wall from limbs with primary venous insufficiency CEAP Class 2 suggest that lesions observed in various stages of venous insufficiency may be associated with an inflammatory process.[1,2] This inflammatory process is includes early leucocyte attachment and infiltration into the valve tissue and this ultimately leads to fibrosclerotic remodelling of the valves and weakening of the vein wall.[3] Haemodynamic forces such as venous hypertension and modified conditions of shear stress in the veins appear to play an important role in triggering this inflammatory reaction. The reaction itself is characterised by early leucocyte activation.[4–6] This, in turn is accompanied by synthesis and release of many inflammatory molecules, including proteolytic enzymes, and possibly other classes of inflammatory regulators.[7–11] The inflammatory reaction perpetuates itself, leading to elongation and tortuosity of the affected veins with splitting, perforation, tearing and ultimate destruction of the venous valves.[12,13] In addition, activated inflammatory mediators and endothelial cell activation may degrade extracellular matrix constituents by release of oxygen free radicals and matrix metalloproteinases (MMPs) activity.[14] Venous hypertension induces morphological changes in valves, including fibrosis, with strong MMP-2 and MMP-9 activity in valves and during remodelling.[15]

In light of these mechanisms for venous remodelling, therapy in the future may have to be directed against the fundamental trigger mechanism, including

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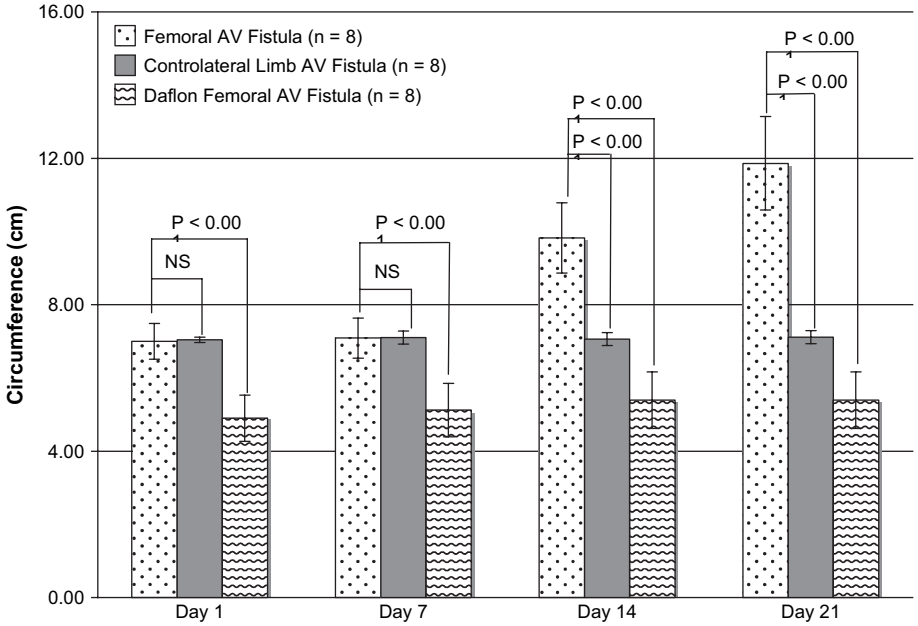

Fig. 1. Hind limb circumferential length (1 cm above the knee) as a function of time (in days) after placement of an AV fistula in the femoral vein. Controls refer to non-pressurised saphenous veins. MMPF (Daflon) treatment was administered over the same period of time (100 mg/kg/day, p.o.) (n = number of animals).

leucocyte and endothelial cell activation.[16] The aim is to diminish cell activation and the magnitude of the inflammatory response.

Daflon® 500 mg, a micronized purified flavonoid fraction (MPFF, S05682, Servier, Paris, France) consisting of 90% diosmin and 10% hesperidin, is used clinically to treat venous insufficiency and haemorrhoidal disorders.[17,18] MPFF has been shown to reduce the expression of adhesion molecules[5,7,19] and the adhesion of leucocytes to the endothelium.[20,21] Recently, it has been shown in a model of rat venous hypertension that treatment with MPFF before elevation of

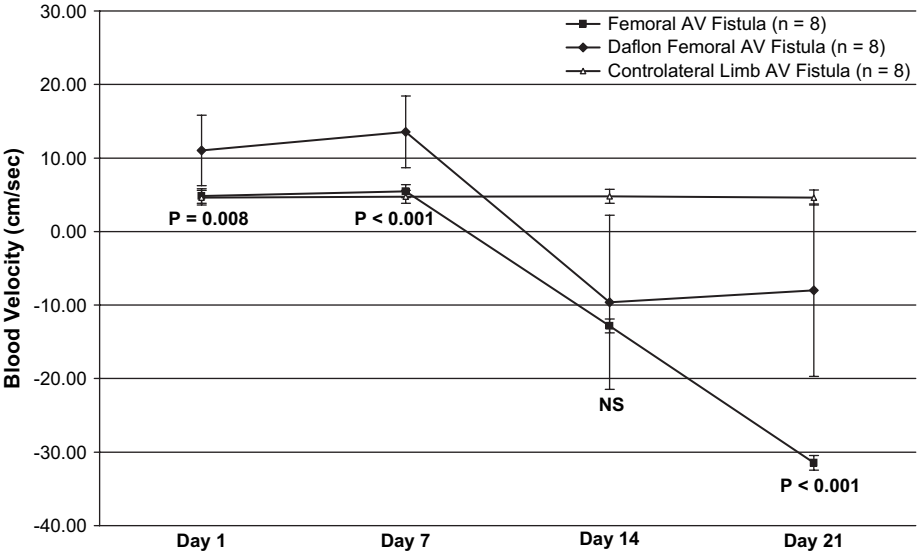

Fig. 2. Peak systolic velocity in the sapheno-femoral junction in the AV fistula group with and without MMPF (Daflon, 100 mg/kg/d) at selected times after placement of an AV fistula in the femoral vein. Positive values refer to forward flow through the saphenous vein and its valve, negative values indicate retrograde flow, reflux through the valves and valve leaflet failure.

venous pressure serves to reduce venous reflux and the shortening of valve leaflets width.[22]

Thus the objectives of the current study were to characterise the response of the vein wall to venous hypertension with its associated altered fluid shear stress and to test the effect of MPFF on this response. We present measurements of limb circumference, blood flow at the saphenofemoral junction under the influence of hypertension generated by an arterio-venous fistula together with measurements of leucocyte infiltration of the sapheno-femoral junction and the magnitude of gelatinase activity in the most affected tissues.

Methods and Materials

Animals

The animal procedures in this study were reviewed and approved by the Animal Subjects Committee of the University of California San Diego. Male Wistar rats (250−300 gm, Charles River Laboratory, Inc., Wilmington, MA, $n = 48$) were operated on under general anaesthesia (pentobarbital sodium, 50 mg/kg, i.m) without tracheotomy in order to facilitate spontaneous respiration.

Arterio-venous fistula

A femoral arterio-venous fistula (AVF) was placed unilaterally in the groin using sterile microsurgical techniques, as described previously.[15,23] Briefly, monofilament sutures (9−0 to 12−0, Ethicon) were used to create a 0.5 mm fistula between the femoral artery and vein proximal to the sapheno-femoral junction.

To minimize venous return and avoid cardiac failure after placement of the AVF, tributaries to the femoral vein including the superficial epigastric vein near the anastomosis were ligated. To prevent blood coagulation, heparin was administered (1000 μ/kg B.W.). The skin incision was closed and the rats were given intensive post-surgical care. Twenty-four rats were housed in a light-cycle controlled flow hood cages and maintained on standard pellet diet and water ad libitum. A group was sacrificed at day 1, a second group at day 7 and a third group at day 21 ($n = 8$ rat in each group). This time period was selected following a series of pilot studies that showed significant dysfunction in the first valve of the sapheno-femoral junction (SFJ) distal to the fistula during this 21-day time interval and ultimately total valve destruction at 42 days. The presence of the fistula raised the blood pressure at the first valve distal to the femoral vein within

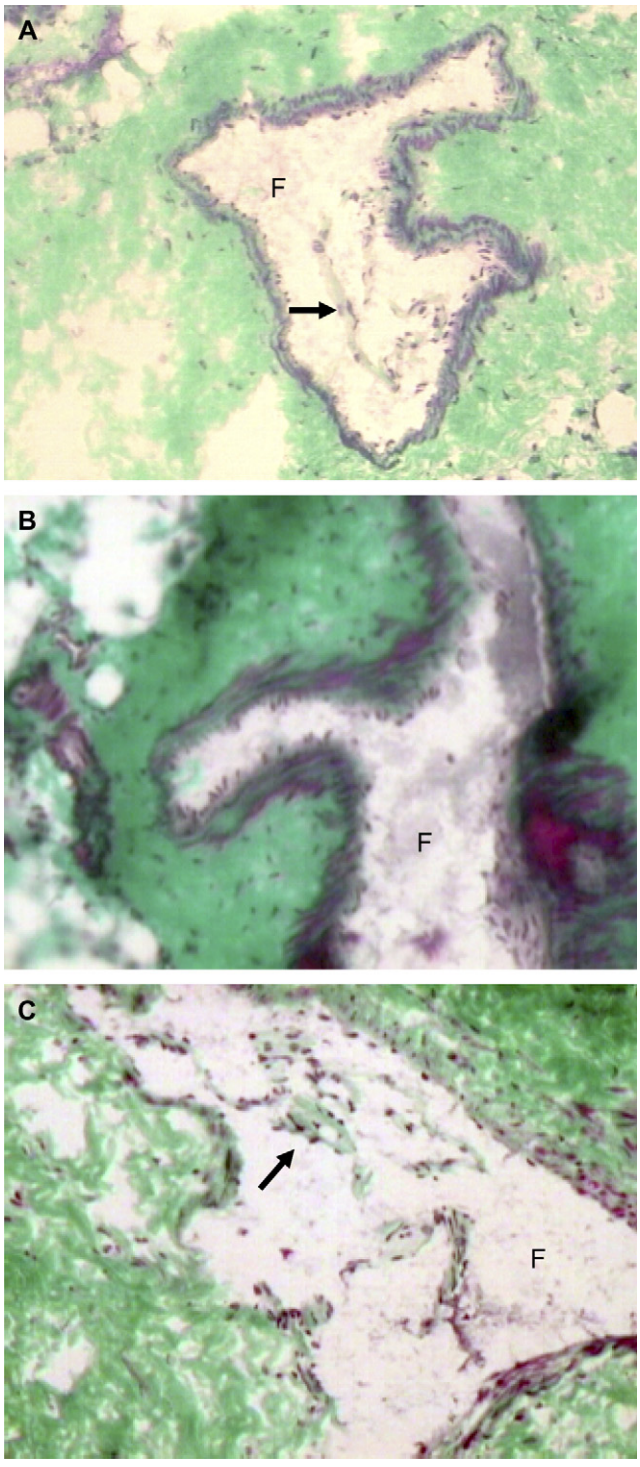

Fig. 3. Micrographs of selected histological sections (Gomori Trichrome stain) through the femoral-saphenous venous junction in (panel A) control animals, and after 21 day placement of AVF (panel B) without and (panel C) with MPFF treatment. Not the absence of any leaflet structure (arrows) in the vessel lumen after exposure to chronically elevated venous pressure (Panel B). Interstitial collages is labelled green, cell nuclei red-purple, venous wall in green-blue.

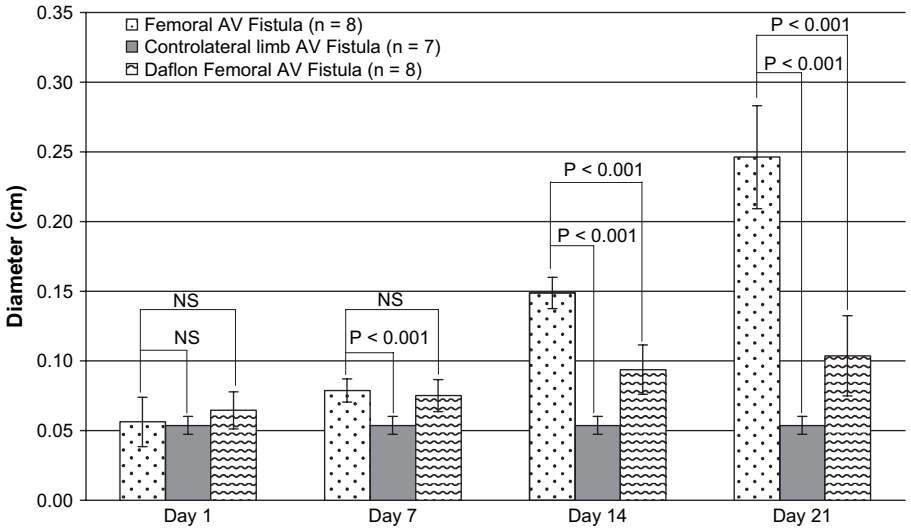

Fig. 4. Venous diameter at the location of the saphenous-femoral valve over the period of time of AVF placement in AVF group, contralateral controls and in MPFF (Daflon) treated group. (n = number of animals).

about 90% of the femoral arterial pressure.[15,23] The contralateral veins served as controls.

Anti-inflammatory treatment

A cohort of twenty-four rats was given an anti-inflammatory treatment with MPFF (Daflon© 500, Servier, Paris, France, 100 mg/kg/day body weight) using an animal feeding tube. Treatment was started 4 days prior to placement of the AVF and continued until completion of the study. Two groups of animals were formed. Group 1 consisted of animals with a femoral AVF without anti-inflammatory treatment, and Group 2 consisted of animals with femoral AVF and treatment with MPFF. The venous tissue was collected under general anaesthesia (pentobarbital sodium, 50 mg/kg, i.m.) for analysis at day 1, 7 and 21 after placement of the AVF ($n = 8$ rats in each group). The contralateral veins served as controls.

Venous reflux examination

At the end of each time period, Duplex Ultrasound examination was carried out in each animal under general anaesthesia (pentobarbital sodium, 50 mg/kg i.p.). The ultrasound system (Philips HDI 5000, Philips Medical Systems, Bothell, WA) was used with a 7–15 MHz hockey-stick transducer. Transverse and longitudinal scans of the femoral venous system of both lower limbs were made. The examination included imaging of the fistula, femoral artery and sapheno-femoral junction. Blood velocities were measured in sapheno-femoral junction 0.5 cm distal to the terminal valve. The diameters of the terminal valve annulus were recorded.

Immunohistochemistry

Specimens from the sapheno-femoral junction were freshly frozen in liquid nitrogen and stored at −70 °C until examination. Longitudinal sections (7 μm thickness) were cut across the valve leaflets with a cryostat at −23 °C, air dried for 30minutes, and fixed in purified acetone for 10 minutes.

Terminal valve morphology was analyzed by the Gomori Trichrome stain on the fixed sections. Leucocyte infiltration was determined by labeling the section with monoclonal antibodies against granulocytes (mouse anti-Rat Gran/Erythroid cells, MCA967, Serotec, Raleigh, North Carolina), macrophages/monocytes (mouse anti-rat CD68, MCA341 R, Serotec), T-lymphocytes (mouse anti-rat CD3, MR5300, Caltag Laboratories, Burlingame, CA), B-lymphocytes (mouse anti-rat CD45RA, MR6400, Caltag Laboratories). Secondary antibodies with fluorescent label (Alexa Fluor 488, Goat Anti-Mouse, A21042, Invitrogen Carlsbad, CA; Alexa Fluor 594 goat anti-mouse, A21125, Invitrogen) were used. Prior to cover-slipping, Prolong Gold with DAPI (P363931; 4′,6-diamidino-2-phenylindole dihydrochloride; Invitrogen) was applied to enhance fluorescence preservation.

The sections were examined with an inverted fluorescent microscope (Olympus IX70, Olympus Center Valley, PA) with 3 filter sets (green 495/519 nm; red ~590/617 nm; blue DAPI 330–380 nm) at objective magnifications between 10X and 60X. Images were

captured with a color couple charge camera, digitized on a laboratory computer (Powerbook G4; Apple Computer Inc. Cupertino, Ca) and analyzed using NIH Image Software (National Institute of Health, Bethesda, Maryland).

In situ zymography

Gelatinase activity was detected in situ by visualisation of fluorescence from the proteolytic cleavage of intramolecularly quenched fluorescein isothiocyanate (FITC)-labeled DQ-gelatin (Molecular Probes). Briefly, 7 µm frozen tissue sections were equilibrated at room -temperature and overlaid with a 20 µl solution containing 20 µl/1 FITC DQ-Gelatin in 20 mmol/L NaCl, 5 mmol/L CaCl2, 0.2 mmol/L NaN3, 0.02% Brj35, and 0.5% low melting point agarose. The sections were then cover-slipped, allowed to gel for 5minutes at 4 °C and incubated for 24 hours at 37 °C. The sections were incubated in presence of activation buffer (100 mM NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM CaCl2, 20 mM ZnCl2, and 0.05% w/v Brij 35 detergent). A part of the sections were incubated in the presence of the metallo-proteinase inhibitor ethylenediaminotetracetate (EDTA, 10 mmol/L). The sections were examined with an inverted (green filter, 495/519 nm) fluorescent microscope at several objective magnifications up to 60X. Images were recorded digitally and analyzed (NIH Image Software, National Institute of Health) on a laptop computer (Powerbook G4; Apple Computer Inc. Cupertino, Ca). Gelatinase activity was determined by fluorescent intensity measurements in three different regions: the femoral vein, sapheno-femoral junction, and the great saphenous vein.

Statistics

Measurements are presented as mean and standard deviation. For each parameter, descriptive statistics are provided at each time by treatment group. Differences between treatments were studied at each time using a two-sided Student's t test for independent samples. $p < 0.05$ was considered to be statistically significant.

Results

Tissue oedema and blood velocity

Limb oedema, as measured by circumferential length 1 cm above the knee joint, progressively increased over the 21-day observation period in the group AVF when compared to control contralateral group with a significant difference at day 14 and day 21 ($p < 0.001$). In the group treated with MPFF, the circumference did not increase between day 1 and day 21 compared to the AVF group ($p < 0.001$) (Fig. 1).

Using ultrasound measurements, the average blood velocity (cm/sec) in the sapheno-femoral junction was markedly increased at 14 and 21 days in the AVF group when compared to contralateral controls without AVF ($p < 0.001$). The group treated with MPFF showed increased sapheno-femoral reflux compared with controls at day 21 ($p = 0.009$), but this reflux was less than in the AVF group at day 21 ($p < 0.001$) (Fig. 2).

The development of the reflux at 21 days indicates loss of valve function at the sapheno-femoral junction, which was confirmed by selected histological sections

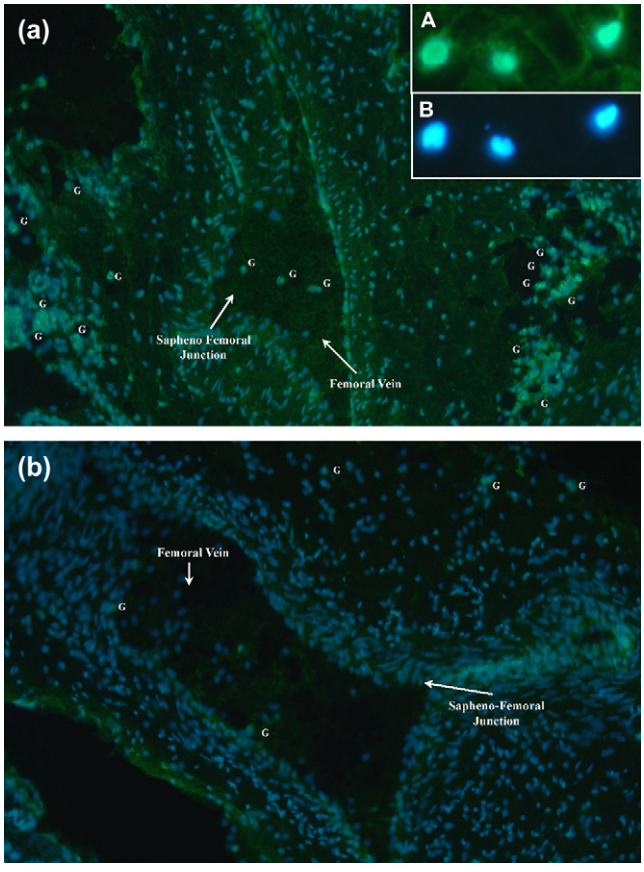

Fig. 5. Micrograph of immunohistological section through sapheno-femoral junction after 21 days of exposure to elevated blood pressure due to the AV fistula (a) without and (b) with MMPF treatment. The sections show fluorescently labelled antibody against granulocytes (G) superimposed on DAPI labelled cell nuclei (insert B in blue colour). Granulocytes were identified by requirement for superposition of the green and blue fluorescence (insert A). Note attenuation of the infiltration by the MPFF.

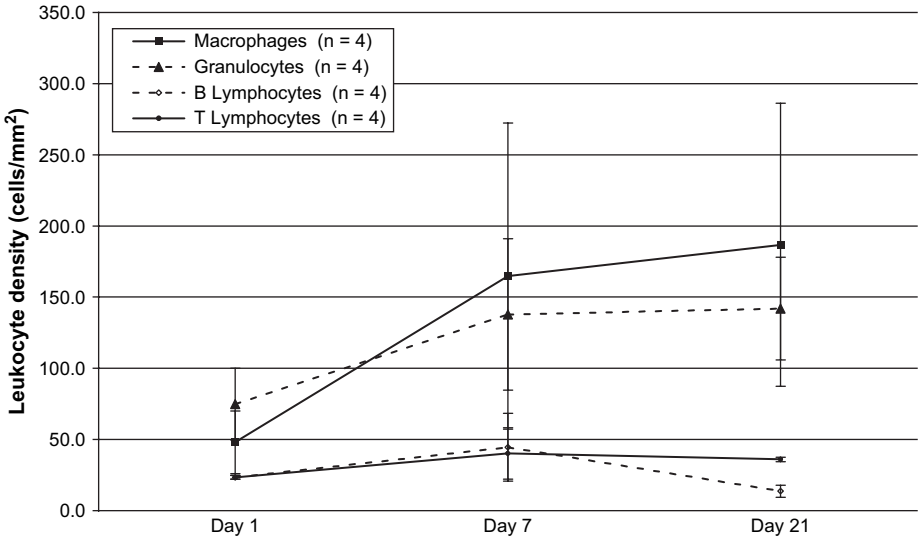

Fig. 6. Time course of leucocyte (macrophages, granulocytes, B-,T-lymphocytes) infiltration in the sapheno-femoral junction after placement of an AV fistula. The cell number is expressed per tissue area (mm^2). (n = number of animals).

showing the absence of any valve leaflets (Fig. 3). Valve leaflets were observed after 21 day in the presence of MPFF treatment.

Valve diameter

The valve diameter increased between day 7 to day 21 in the AVF group when compared to controls ($p < 0.001$). The MPFF treatment group had a slower increase in diameter with less augmentation compared to the AVF group animals at day 14 and day 21 ($p < 0.001$) (Fig. 4).

Leucocyte infiltration

In the tissue of the AVF group surrounding the sapheno-femoral junction and a small part of the femoral vein, there was over the period of 21 days a progressive increase in granulocyte infiltration ($p = 0.023$ between day 1 and day 21) (Figs. 5a, 6). In this group, a plateau of infiltration was reached at day 7 which then persisted but did not increase further. In the AVF group an increase in the number of macrophages/monocytes was seen between day 1 and 7, followed by a plateau until day 21 (Fig. 6). We saw an increase of T lymphocytes (by about 37%) ($p < 0.001$ between day 1 and day 21 but only a small non-significant increase in B-lymphocyte infiltration in the sapheno-femoral junction at day 1 and 7 in the AVF group.

Compared with the untreated AVF group, treatment with MPFF decreased granulocyte and macrophage infiltration into the sapheno-femoral parenchyma at each time point (p values between 0.028 and 0.004 for granulocytes; p values between 0.022 and 0.001 at day 1 and day 21 for marcrophages) (Figs. 5b, 7). Treatment with MPFF decreased B-lymphocyte infiltration only at day 1 compared to the untreated AVF group (results not shown).

In situ zymography

In the AVF group, the gelatinolytic activity (MMP2 and MMP9) in the femoral vein (Region 1), the sapheno-femoral junction (Region 2), and greater saphenous vein (Region 3) was enhanced on days 1 ($p = 0.016$ region 1, $p = 0.001$ region 2), 7 ($p < 0.001$ region 1, $p = 0.073$ region 2, $p = 0.001$ region 3) and 21 ($p < 0.001$ region 1, $p < 0.001$ region 2, $p = 0.005$ region 3) as compared to contralateral controls (Fig. 8). The activity was blocked below detection limit in the presence of the metal chelator EDTA (Fig. 8). Treatment with MPFF did not inhibit gelatinolytic activity in the different sites (results not shown).

Discussion

While the A-V fistula model is not a perfect representation of human venous hypertension, it is the only chronic venous hypertension that allows serial observations over time. Placement of a femoral AVF causes stretch of the venous wall due to a significant pressure elevation and likely also changes the fluid shear stress on the endothelium in the venous vasculature of the

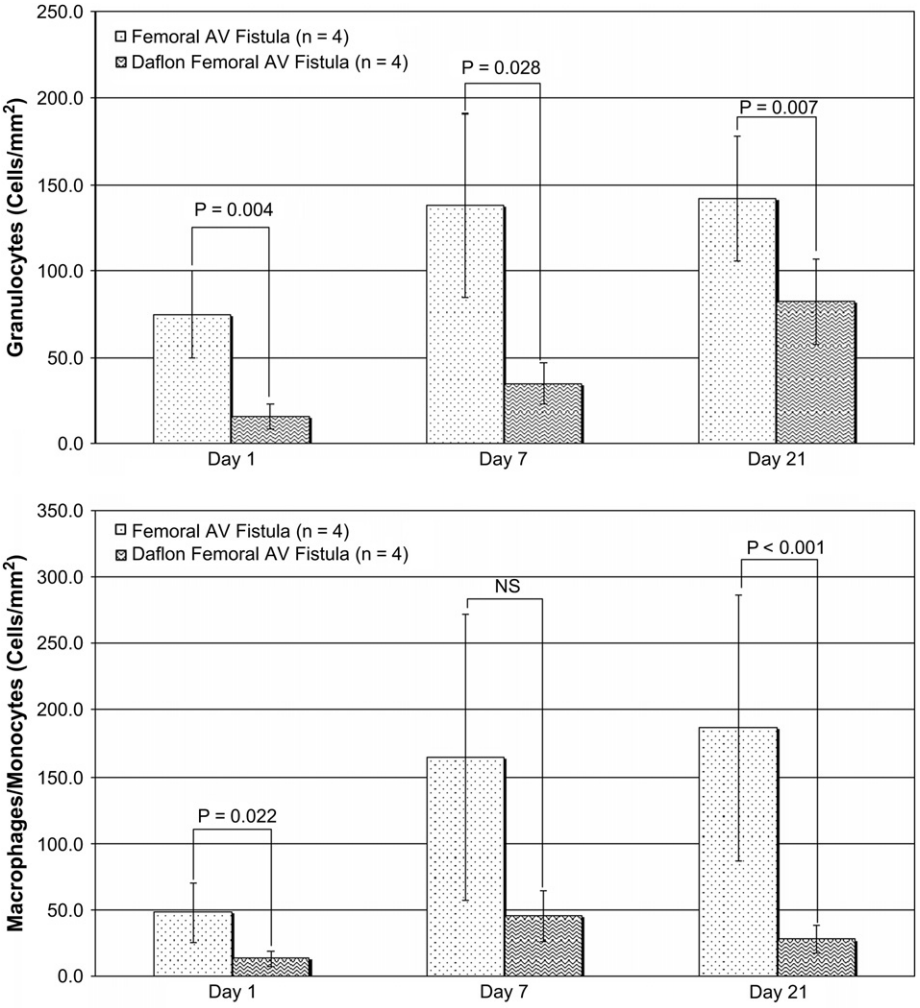

Fig. 7. Time course of granulocyte (top graph) and macrophage/monocyte (bottom) density (number of cells per tissue area, mm^2) into the sapheno-femoral junction without and with MPFF (Daflon) treatment after placement of an AV fistula. (n = number of animals).

limb. This in turn is accompanied by limb edema and an increase in limb circumference together with an eventual loss of valve leaflets in the saphenous vein. There is an increase in granulocyte and macrophage infiltration into the wall and the surrounding tissue and a lesser but still detectable increase in T- and B-lymphocyte infiltration. These changes are all attenuated by oral administration of MPFF. The observations corroborate previous observations regarding venous valve remodelling under the influence of venous hypertension[16] and in addition confirm the ameliorating effect of MPFF on venous insufficiency. The oedema as measured by limb circumference was significantly reduced with preservation of valve function.

The observations in this study suggest that acute venous hypertension in the femoral vein induced by an AV fistula is accompanied by inflammatory markers. Markers of inflammation in form of T-cell, granulocyte, and macrophage infiltration into the vein wall are seen. The situation resembles valve failure and venous insufficiency in man except that in this acute model *granulocyte* infiltration is encountered. In chronic venous insufficiency in man the predominant cell infiltrating the venous valve is the *monocyte*.[2] This lack of neutrophil detection may in part be due to the fact that there is currently no technique in patients to detect neutrophil or any other leucocyte infiltration in-vivo over a prolonged period of time. Instead the analysis has to rely on biopsies obtained at single time points during the progression of the disease. Leucocyte infiltration into the femoral vein and the SFJ as seen in this model is one of the hallmarks of the inflammatory process and in line with previous histological observations in the valves and venous wall of

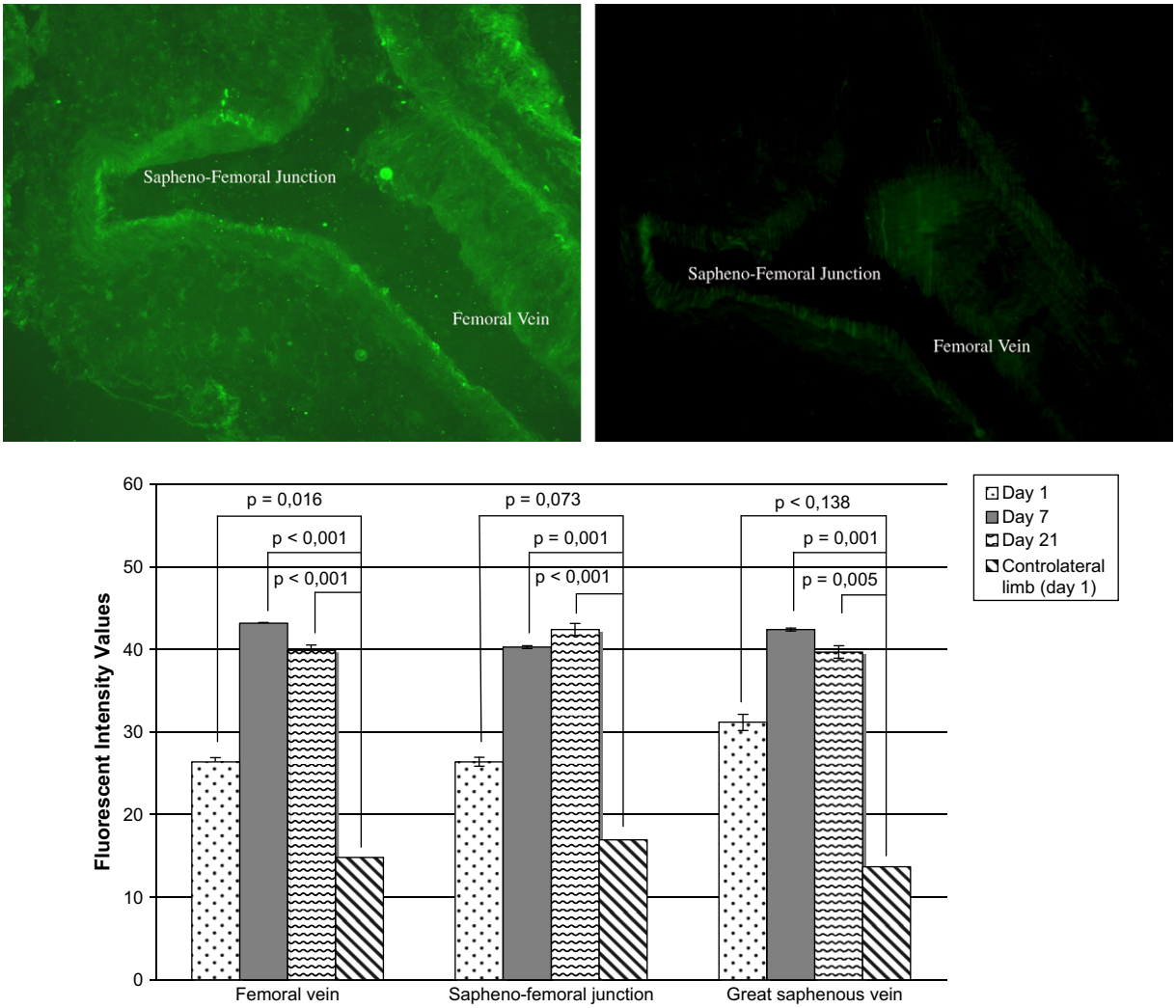

Fig. 8. (Top Panels) In-situ zymographic fluorescent micrographs of femoral-saphenous junction showing the distribution of gelatinase activity (top left panel). It was confirmed to be due to MMPs (MMP-2, -9) by metal chelation with EDTA (top right panel). (Bottom Panel) Average fluorescent intensity (expressed in digital units) due to MMP activity in the wall of the femoral vein (Region 1), the sapheno-femoral junction (Region 2), and the saphenous vein (Region 3) of untreated AVF group. $n = 4$ rats in each group.

patients with venous dysfunction.[2] The response of the leucocytes in man is probably preceded by a set of separate inflammatory events in the acute phase of venous pressure elevation, as seen in the present model but the acute phase and its preliminary events are currently not capable of being studied.

The current model represents a severe form of venous pressure elevation. Likely, the stress-induced inflammation is greater in this model than what is encountered in man. An exception may be at the ankle in man and in perforating veins where the pressures encountered are comparable to those in this experimental preparation.[24] Pressures in perforating veins caused by muscular contraction are comparable to those generated in this model.[24]

In previous studies,[22] we reported expansion of the venous wall under elevated pressure generated by the AVF. In the early stages after placement of the AVF the valve leaflets were still able to close properly without significant reflux. Thus the elevated pressure *per se* is not the only variable that compromises the leaflets although it may produce a progressive expansion of the venous diameter to the point that the length of the leaflets may be insufficient to achieve complete closure. At the time the leaflets fail and reflux occurs, we see a reduction of the leaflet dimensions in a sequence of events that leads to valve destruction, much as is seen in man.[25] Destruction of the valve leaflets may be induced not only by stretch of the venous wall and the leaflets but also by the onset of an abnormal fluid

shear stress on the endothelial surface of the leaflets. As the valve ceases to close completely a reversed shear stress may arise during venous reflux. Fluid shear stress on the endothelium, if accompanied by unsteady retrograde (with instances of reversed shear direction)[26] or even turbulent (with random fluctuation) stress,[27] may be an inflammatory stimulus for the endothelial cells on a valve leaflet and may trigger cell apoptosis, degradation of the collagen matrix of the valve, and consequently mechanical softening and restructuring of the leaflets and the venous wall.

MPFF administration reduced the oedema, the valve annulus diameter, and fistula blood flow produced by the acute AVF in line with its ability to block elevation of microvascular permeability in inflammation.[28,29] MPFF also reduces granulocyte and macrophage infiltration in line with observations in experimental acute ischemia and reperfusion or ischemia produced by venous hypertension.[21] The current AVF model brought to light the ability of MPFF to attenuate infiltration into the vein wall and valves as well. These activities by a combination of flavonoids are in line with the hypothesis that an early inflammatory signal is being blocked. The exact nature of this signal remains to be identified. Meanwhile our observation suggest that blocking the remodelling of venous valves and the vein wall in venous insufficiency in man may serve to shift the therapeutic approach from treatment to an earlier stage of intervention.

Acknowledgement

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